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Interferon Antagonism as a Common Virulence Factor of Hemorrhagic Fever Viruses

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Abstract:
We examined the ability of viruses in the *Hantavirus* and *Nairovirus* genera of the family *Bunyaviridae* to interfere with host signaling pathways involved in innate immunity. For the nairovirus, Crimean Congo hemorrhagic fever virus (CCHFV), we found that the viral polymerase gene contains a predicted ovarian tumor (OTU) protease domain that functions to deconjugate ubiquitin and interferon stimulated gene product 15 (ISG15) from host proteins. Both ubiquitin and ISG15 reversibly conjugate to proteins via a conserved LRLRGG C-terminal motif, mediating important innate antiviral responses. We showed that the OTU domain-containing protease of CCHFV hydrolyzes ubiquitin and ISG15 from many cellular target proteins. This broad activity contrasts with the target specificity of known mammalian OTU domain-containing proteins. The biological significance of this activity of viral OTU domain-containing proteases was evidenced by their capacity to inhibit nuclear factor kappa B (NF-κB) dependent signaling and to antagonize the antiviral effects of ISG15. The deconjugating activity of viral OTU proteases represents a novel viral immune evasion mechanism that inhibits ubiquitin- and ISG15-dependent antiviral pathways. For the hantavirus, Hantaan virus (HTNV), we found that the nucleocapsid protein was able to inhibit tumor necrosis factor alpha (TNF-α)-induced activation of NF-κB as measured by a reporter assay and activation of endogenous p65, a NF-κB subunit. We showed an interaction between HTNV N protein and importin-α, a nuclear import molecule responsible for shuttling NF-κB to the nucleus. These data suggest that HTNV N protein can sequester NF-κB in the cytoplasm, thus inhibiting its activity.

INTRODUCTION: The overarching goal of this project is to identify common mechanisms that hemorrhagic fever viruses use to evade host innate immune responses and to develop means to overcome those evasion strategies. Among the viruses in our study are representatives of three different genera of the family *Bunyaviridae*: Hantaan (HTNV), Rift Valley fever (RVFV), and Crimean Congo hemorrhagic fever (CCHFV). In addition, we are studying members of the *Filoviridae* and *Arenaviridae* families: Ebola virus (EBOV) and Lassa virus (LASV), respectively. Our approach and goals are to (1) determine if the viruses evade host innate immunity; (2) to identify viral genes and proteins involved in immune evasion; (3) elucidate the mechanism(s) by which evasion occurs; and, (4) search for therapeutics that are not susceptible to those mechanisms. Studies from the first year centered on CCHFV and hantaviruses. These viruses have three-segmented RNA genomes (S, M, L), which encode the nucleocapsid N protein, glycoproteins (Gn and Gc), and the transcriptional polymerase (L), respectively in the virus complementary RNA. Our studies indicate that the L segment of CCHFV and the S segment of hantaviruses are used in infected cells to evade host innate immunity through novel and previously unknown mechanisms of action.

FINDINGS:

CCHFV possesses a novel evasion strategy that is mediated by the viral polymerase gene
We identified a unique domain in the polymerase gene of CCHFV that has ubiquitin deconjugating activity. As ubiquitination is a key factor in signaling transcription factors needed to trigger innate immunity, the presence of this domain allows CCHFV to interfere with the induction of at least one pathway in the host response to viral infection. The results of that study were published (manuscript is appended), and detailed methods and specific results are presented therein. As described in the manuscript, we showed that a viral ovarian tumor (OTU) domain is present in the L gene of CCHFV and

that it functions as a protease able to hydrolyze ubiquitin and interferon stimulating gene product 15 (ISG15) from conjugated proteins. This protease activity provides the physiologic capacity to evade two different cytokine pathways, IFNαβ and TNFα, which are fundamentally important for innate immunity. Viral OTU domain proteases inhibit protein ISGylation in order to counter some of IFNαβ's antiviral effects, while viral deubiquitinating activity prevents TNF-α transcriptional effects. This dual deconjugating activity provides an elegant example of the economy of viral evolution since both ubiquitin and ISG15 rely on a conserved LRLRGG motif that is essential for their conjugation. By targeting this conjugation process, viruses can evade or subvert many different cellular processes.

Although OTU domains are present in mammalian proteins, we found that the OTU domain-containing cellular proteins A20, Cezanne, Otubain 1, Otubain 2 and VCIP do not exhibit the unique capacity of the CCHFV OTU domain protease to target both ubiquitin and ISG15 conjugates. We speculate that other viral proteases, perhaps including some that do not have clear OTU domains, will be found to target both ubiquitin and ISG15 dependent processes. We further speculate that the OTU domain specificity and deconjugating activity provides a unique target for antiviral drug development. The characterization of the CCHFV-L OTU domain and the development of in vitro assays for its enzymatic activities as described in this study will make it feasible to screen for potential inhibitors specific for CCHFV-L and other OTU domain-containing viral proteins.

Hantaan virus (HTNV) nucleocapsid (N) protein interferes with tumor necrosis factor alpha (TNF-α)-signaling for the induction of the NF-κB.

The results, as described below, are currently being reviewed internally for submission to J. Virology.

TNF-α is a major proinflammatory cytokine produced by a variety of cell types that include macrophages, endothelial cells and epithelial cells and the receptor is constitutively expressed on most cell types (26). TNF-α is pleiotropic and can regulate the response of immune cells as well as induce inflammation, differentiation, apoptosis, and is involved in protecting the host from pathogen infections. Upon TNF-α ligand binding, TNF-associated death domain (TRADD) associates with the TNF receptors (TNFR) and serves as an adaptor molecule that recruits TNFR-associated factor-2 (TRAF-2) and receptor interacting protein (RIP) (10). This complex of proteins leads to the activation of kinases that phosphorylate inhibitor of κB (IκB) (10). The ubiquitin-proteosome pathway initiates degradation of IκB allowing for NF-κB dimers to translocate to the nucleus and regulate transcription of its target genes.

NF-κB transcription factors are dimers composed of five subunits belonging to the Rel family (20). The five subunits of p65 (Rel A), Rel B, c-Rel, p50, and p52 can form various dimers (2, 23, 24). The p50/p65 heterodimers are the best characterized and are the most abundant form of the NF-κB transcription factors in most cell types (8). IκB is responsible for sequestering NF-κB in the cytoplasm by masking its nuclear localization signal (NLS) (3, 6, 9, 27). Proteins that require nuclear translocation contain a NLS and are transported by importin α via its' interaction with the cargo's NLS (7, 16). To date there have been six importin α family members identified (importin α1-6), all of which carry various cargo, including signal transducers and activators of transcription (STATs) and NF-κB (4, 11, 12, 17, 21). Importantly, it was recently reported that TNF-α-induced nuclear localization of p50/p65 heterodimers is mediated by importin α3 and importin α4 (5). However, there have also been reports of importin α1 and importin α2 interacting with NF-κB, but their role in transport and activation remains unclear (5). Because of the multifaceted nature of TNF-α and NF-κB, the NF-κB subunits and the importin α proteins have become prime targets of viruses to evade the outcome of inflammatory pathways.

Hantaviruses can cause two distinct types of human disease: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (19). Hantavirus associated diseases are thought to be immunologically mediated and there have been numerous reports of patients having elevated plasma levels of TNF-α during the acute phase of HFRS (13-15). TNF-α positive cells can also be found in kidney and lung biopsies of HFRS and HPS patients. Clinically, hypotension and patient outcomes correlate with the levels of TNF-α (14, 15).

Recently, studies, in addition to ours, suggest that hantaviruses can subvert innate immunity through several mechanisms. The Gc protein of the HPS-causing viruses, Andes virus (ANDV) and NY-1 virus (NY-1V) can inhibit activation of two important innate immune pathways, double stranded RNA (dsRNA) and interferon (IFN). NY-1V Gc has been shown to antagonize the activation of RIG-I, a protein involved in the induction of IFN (1). ANDV Gc can inhibit the phosphorylation of transcription factors STAT1 and STAT2 and this was also shown in HTNV infected cells (22). Furthermore, another HPS-causing hantavirus, Sin Nombre virus, was also unable to induce production of IFN-α/β during infection (18). Considering the clinical relevance of TNF-α during infection, we sought to determine if HTNV might also interfere with this inflammatory pathway.

HTNV N protein inhibits TNFα-induced activation of NF-κB. To date there have been no reports of studies examining the ability of HTNV to antagonize the TNF-α signaling pathway. To determine if the HTNV N, Gn, or Gc proteins could inhibit TNF-α-induced activation of NF-κB, a human NF-κB promoter that transcriptionally regulates the expression of GFP was used as a reporter. No GFP was detected in any of the samples that were left untreated indicating that the conditions of our experiment alone, including the presence of viral proteins, could not induce activation of NF-κB (Fig. 1A and B). HTNV N protein was able to inhibit transcriptional GFP expression from the NF-κB responsive promoter at all concentrations of TNF-α when compared to empty vector (Fig 1A). HTNV glycoproteins only had little effect on GFP expression suggesting that this phenomenon is N protein specific (Fig. 1A). This inhibition occurred in a dose dependent manner but was still significantly less than the control empty vector (Fig 1A).

Proteosomal degradation of IκBα is required for the activation of NF-κB in the TNF-α pathway. In order to validate our assay, the proteosomal inhibitor MG132 was included as a control because it has been previously shown to inhibit this pathway. MG132 was also able to dramatically decrease the amount of GFP present in TNF-α treated cells and this was comparable to what we observed in cells expressing the HTNV S segment cDNA (Fig. 1A).

We also examined whether TNF-α-induced activation of our reporter system correlated with the amount of viral protein or MG132 present in cells. As expected, increasing amounts of empty vector and HTNV glycoproteins had no effect on the expression of the NF-kB-GFP reporter (Fig. 1B). However, inhibition of NF-kB- GFP was directly proportional to the amount of HTNV nucleocapsid and MG132 in cells, hence the greatest inhibition was observed with maximal N protein and MG132 present (Fig. 1B). It should also be noted that expression of the HTNV N and glycoprotein genes were not adversely affected by the amount or time that cells were treated with TNF-α (data not shown).

HTNV N protein inhibits activation of endogenous NF-κB p50 and p65. In order to validate the NF-κB reporter assay used above, we next examined whether HTNV N protein was able to inhibit activation of endogenous p50 and p65 subunits, the most abundant of the heterodimers used as an NF-kB transcription factor. To quantify NF-kB transcription factor activation, we employed an ELISA that measures binding to NF-kB consensus oligonucleotide sequences, followed by detection of p50 and p65 subunits with antibodies. As expected, there was little activation of transfected and untreated cells indicating that viral gene expression and transfection alone could not activate NF-kB proteins (Fig. 2A). In contrast, similar levels of activated p50 and p65 were detected from nuclear extracts of TNF-α stimulated cells that were transfected with empty vector or HTNV-M cDNA (Fig 2A). Transfecting with HTNV -S cDNA significantly inhibited activation of p50 and p65 as determined with extracts of TNF-α stimulated cells and this was similar to what we observed with MG132 treated cells as compared to cells transfected with the empty vector (Fig. 2A). Although there was a small increase in activation of p50 and p65 in HTNV-S expressing cells treated with TNF-α as compared to untreated cells, this can most likely be explained by the fact that we were measuring activation of endogenous NF-kB in a total cell population, but not every cell was expressing the HTNV genes during the period of assay.

To confirm that cells were expressing viral genes, we prepared cytoplasmic extracts and examined them by immunoblotting to detect N protein (Fig. 2B) or by immunofluorescent antibody staining to detect Gn

and Gc (Fig. 3). Levels of the housekeeping protein GAPDH were also measured to rule out the possibility that the decreased inhibition we observed for HTNV N protein and MG132 treatment was not due to differences in the amount of lysate used in our assay (Fig. 2B).

HTNV nucleocapsid inhibits TNF-α -induced nuclear translocation of NF-κB p65. The transcription factor ELISA used above is not able to distinguish between inhibition due to reduced DNA binding or to the inability of NF-kB to enter the nucleus. To test whether cellular localization was altered in TNF-α-treated cells expressing HTNV -S, we performed an immunofluorescence assay. When localization and expression of p65 was examined by microscopy, all unstimulated and transfected or MG 132-treated cells had the expected cytoplasmic localization with no apparent differences in p65 protein expression within cells (Fig 3, upper panel). In contrast, nuclear translocation of p65 was observed in cells transfected with empty vector or HTNV-M cDNA and treated with TNF-α (Fig. 3, lower panel, 1st and 3rd column). This indicates that there was no defect in the ability of p65 to move to the nucleus nor could transfection and viral gene expression alone inhibit this event. In contrast, TNF-α cells that were expressing HTNV-S cDNA had markedly reduced p65 that translocated to the nucleus (Fig. 3, lower panel, 2nd column). As expected, treatment of cells with MG132 was also able to inhibit p65 from entering the nucleus (Fig. 3, lower panel, 4th column). These data suggest that p65 is being sequestered in the cytoplasm by HTNV-N protein.

HTNV–S expression does not affect protein levels of p50 and p65 or degradation of IκB. Since it was difficult to definitively determine by microscopy whether the decreased nuclear p65 observed was due to retention in the cytoplasm or a decrease in the amount of total p65 present in cells expressing HTNV-S, we performed western blot analysis. No difference in the protein levels of p50 and p65 were observed in any of the samples that were transfected and unstimulated or stimulated with TNF-α (Fig. 4). This indicates that the observed decrease in nuclear localization of p65 was probably due to the inability of NF-κB to enter the nucleus. β-Actin was used as a control to ensure equal loading of samples.

The TNF-α pathway requires the association of numerous signaling molecules such as TRADD, TRAF-2, and RIP and alteration of any of these molecules could inhibit activation of NF-κB (25). The next question we wanted to address was whether the NF-κB proteins were being targeted directly by HTNV N protein or if a signaling molecule further upstream was being inhibited thus preventing activation of NF-κB. Since IκBα serves as an inhibitor of NF-κB and its degradation is required for translocation of activated NF-κB to the nucleus, we examined the ability of HTNV N protein to inhibit degradation of IκBα in the presence of TNF-α. Western blot analysis of IκBα levels demonstrated that TNF-α treatment was effective in subsequent degradation of IκBα in all transfected samples including HTNV–S segment expressing cells (Fig 4). Furthermore, IκBα levels were similar in all unstimulated and transfected samples (Fig 4).

HTNV N protein binds to nuclear import proteins, importin α. The above data suggest that NF-κB is being directly sequestered by HTNV N protein or has lost its ability to translocate to the nucleus. To examine if NF-κB was directly targeted we performed co-immunoprecipitation experiments to determine if HTNV N protein could interact with p50 or p65. No interaction was detected between HTNV N protein and NF-κB proteins nor did we detect any inhibition of the phosphorylation of NF-κB in the presence of HTNV N protein when treated with TNF-α (data not shown). These results suggest that transport of NF-κB was likely inhibited by HTNV N protein.

It has been reported that NF-κB accumulates in the nucleus via interaction of importin α3 and importin α4 and its nuclear localization signal. We next wanted to ask whether HTNV nucleocapsid could interact with these or possibly other importin α molecules. FLAG-tagged importin α1, importin α2, importin α3, or importin α4 were co-transfected with empty or HTNV-S expression plasmids. Immunoprecipitation with an anti-HTNV-N protein polyclonal antibody followed by western blotting with anti-FLAG antibody was used to detect importin α proteins. The results indicate that HTNV N protein is able to interact with importin α1, importin α2, and importin α3 and that the presence of TNF-α is not required for this interaction (Fig. 5). Western blotting of whole cell lysates demonstrated that there were similar levels of importin α and nucleocapsid protein in our samples.

HTNV inhibits TNF-α-induced nuclear localization of NF-κB p65 without affecting IκBα degradation. The HTNV S segment transfection experiments clearly demonstrated inhibition of NF-κB. To confirm that infection with HTNV also inhibits TNF-α-induced activation of NF-κB by preventing nuclear localization, we examined virus infected cells by immunofluorescent antibody staining with antibodies to p65 and HTNV N protein. We did not detect nuclear p65 in unstimulated cells that were mock-infected or infected with HTNV (Fig. 6A, upper panel). As expected, TNF-α stimulated mock-infected cells had predominantly nuclear localization of p65 (Fig 6A, lower panel, 1st column). Interestingly, as infection progressed to day 5, the staining pattern of p65 became predominantly cytoplasmic in TNF-α treated cells and this was similar to that of unstimulated cells (Fig 6A, lower panel, 2nd column).

To confirm that there was no decrease in the total protein levels of p50 or p65 and that no upstream signaling molecules were being targeted, we performed western blot analysis. Mock-infected or HTNV-infected lysates harvested after unstimulation or stimulation with TNF-α had no apparent differences in the overall levels of p50 or p65, indicating that this was not the cause of decreased nuclear localization in the presence of virus (Fig 6B). HTNV was also unable to inhibit degradation of IκBα after being treated with TNF-α, suggesting that IκBα itself and signaling molecules upstream are not being targeted by HTNV (Fig. 6B).

All of the results summarized above were included in two peer-reviewed publications listed as reportable outcomes below:

Other results:

We observed that the HTNV N contains amino acids consistent with a nuclear localization signal (NLS). We hypothesize that this NLS area is responsible for the binding of N to the α importins. Further, we observed that similar amino acid motifs are also present in the N proteins of several other hantaviruses. Therefore, to determine if this feature is a common mechanism for subverting host innate immunity by hantaviruses, we tested importin α binding and down-regulation of NF-κB with additional Old World, HFRS-causing hantaviruses (Seoul and Dobrava viruses) or New World, HPS-causing hantaviruses (Andes, and Sin Nombre viruses). As we predicted, we were able to show that expressed N of all of these viruses both bound to the α importins and interfered with activation of NF-κB. Thus, it appears that we have discovered a novel mechanism that is conserved among hantaviruses for subverting the innate immune response.

In addition to these studies on NF-κB activation, we also began studies to define other innate immune evasion strategies. We focused on antagonism of the Type 1 interferon (IFN) system because it is usually the first line of defense against viral infections. Evasion of the IFN response by viruses can occur by preventing IFN release, inhibiting IFN-signaling and/or activity of IFN-stimulated genes. To date there have been no reports of studies examining IFN antagonism of hantaviruses or of CCHFV. To examine this for hantaviruses, lung epithelial cells (A549) were mock-infected or infected with the HPS-causing hantavirus, Andes virus (ANDV), for 7 days and left untreated or treated with IFN every day. We determined by Real Time RT-PCR and western blot that the ANDV S segment synthesis and nucleocapsid production peak on day 4. We also examined the levels of phosphorylated STAT1 and STAT2 by western blot. In infected samples left untreated, little phosphorylated STAT1 and STAT2 was detected up to 4 days post infection. Beyond 4 days post infection, phosphorylated STATs were not detectable by western blot and immunofluorescence. In samples treated with IFN, ANDV was able to inhibit phosphorylation of STAT1 and STAT2 to the greatest extent starting on day 3 when compared to mock-infected cells. Furthermore, this inhibition was not a result of STAT1 or STAT2 being degraded. These data suggest that hantaviruses have the capability to subvert a host IFN response.

To examine IFN antagonism with CCHFV, 293T or HepG2 cells were mock-infected or infected with CCHFV for 5 days and left untreated or treated with IFN every day. We found that CCHFV activates the transcription factor IRF-3, thus allowing for transcription and production of IFN. The IFN made during infection retains its ability to activate STATs through the IFN signaling pathway and allows for

upregulation of IFN stimulated genes. We did not detect any degradation of PKR, eIF2-alpha, or ISG15, all of which are upregulated by IFN. Although this project has ended, we hope to be able to continue this very interesting line of research, aimed at identifying mechanisms that hemorrhagic fever viruses have developed for evading host immunity.

KEY RESEARCH ACCOMPLISHMENTS:

- CCHFV possesses a novel evasion strategy that is mediated by the viral polymerase gene and results in deconjugation of ubiquitin and ISG15 from host proteins.
- HTNV and other hantaviral nucleocapsid proteins interfere with tumor necrosis factor alpha (TNF-α)-signaling for the induction of the NF-κB by binding to cellular transport proteins and preventing their use in moving TNF-α from the cytoplasm to the nucleus of infected cells.

REPORTABLE OUTCOMES:

1. Frias-Staheli, N., N. V. Giannakopoulos, M. Kikkert, S. L. Taylor, A. Bridgen, J. Paragas, J. A. Richt, R. R. Rowland, C. S. Schmaljohn, D. J. Lenschow, E. J. Snijder, A. Garcia-Sastre, and H. W. t. Virgin. 2007. Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. Cell Host Microbe 2:404-16.
2. Taylor, S.L., N. Frias-Staheli, A. García-Sastre, and C. S. Schmaljohn. Hantaan Virus Nucleocapsid Protein Binds To Importin αProteins And Inhibits Tumor Necrosis Factor-α -Induced Activation Of Nuclear Factor Kappa B. J. Virol. published ahead of print on 19 November 2008.

CONCLUSION:

We discovered two previously unknown mechanisms that hemorrhagic fever viruses use to subvert innate immunity by disrupting NF-κB signaling pathways. For CCHFV, we discovered a domain in the viral polymerase protein with the capacity to inhibit NF-κB-dependent signaling (ubiquitination) and to antagonize the antiviral effects of ISG15 in vivo. For HTNV we showed that the viral nucleocapsid protein binds to cellular transport molecules (importins), preventing the transport of TNF-α into the host cell nucleus. By sequestering TNF-α in the cytoplasm, HTNV prevents its use as a transcription factor to stimulate host genes involved in inflammatory and apoptosis processes. This is especially important since elevated levels of TNF-α are a hallmark of hantaviral diseases. We hypothesized that the ability of HTNV N protein to bind to cellular importins was related to a specific region of the gene that encodes a nuclear localization signal (NLS). We observed that similar or identical NLS sequences are present in the N genes of other hantaviruses, and we showed that the N proteins of these viruses are also able to bind the importin α proteins and to prevent activation of NF-κB. Further studies to determine if this mechanism is also used by other hemorrhagic fever causing viruses are needed. In addition to these two new discoveries, we also demonstrated that the hantavirus, Andes virus, interferes with the in JAK-STAT pathway of interferon induction by inhibiting translocation of STAT1 and STAT2 to the nucleus.

In summary, we made new discoveries concerning how hemorrhagic fever viruses are able to evade host innate immunity. Further studies are needed to determine if these mechanisms are common to several viruses and if they can be overcome by exogenous interferon or other therapeutic means.

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FIGURE LEGENDS:

Fig. 1 The effect of HTNV N protein on NF-κB gene expression. (A) 293T cells were co-transfected with 500 ng of pNF-κB-hrGFP and 500 ng of either pWRG7077-Empty, pWRG7077-HTNV-S or pWRG7077-HTNV-M. After 24 h, cells were incubated in medium with or without TNF-α (0 to 100 ng/ml) for 4 h. As a positive control for inhibition in our assay, cells transfected with only 500 ng of pNF-κB-hrGFP were pretreated with 50 μM MG132 for 2 h before the addition of TNF-α and throughout the experiment. (B) Cells were co-transfected with 500 ng of pNF-κB-hrGFP and 5-500 ng of either pWRG7077-Empty, pWRG7077-HTNV-S or pWRG7077-HTNV-M. Or transfected with 500 ng of pNF-κB-hrGFP and treated with 0-50 μM MG132. After 24 h, cells were incubated in medium with or without TNF-α (10 ng/ml) for 4 h. After 4 h of treatment, medium was removed from all wells and replaced with medium lacking TNF-α for an additional 24 h. GFP expression was examined by fluorescence microscopy.

Fig. 2 Endogenous NF-κB transcription activation in cells expressing HTNV-S segment cDNA. A549 cells were transfected with 500 ng of pWRG7077-Empty, pWRG7077-HTNV-S, or pWRG7077-HTNV-M for 24 h. After treating the cells with 50 ng/ml of TNF-α for 15 min, cytoplasmic and nuclear extracts were prepared from lysates. (A) Nuclear extracts were allowed to bind to NF-κB consensus sequence oligos on 96-well plates followed by probing with antibodies specific for NF-κB p65 or NF-κB p50. Absorbance reading for each sample was determined using a spectrophotometer. (B) Cytoplasmic extracts were used for immunobloting to detect HTNV N protein and GAPDH. Statistical significance of the wild-type oligo+TNF was determined by comparing to the MUT oligo+TNF. Significance of N protein+TNF and MG132+TNF was determined by comparing to empty vector + TNF .

Fig. 3 Intracellular localization of NF-κB p65 in HTNV-S segment expressing cells. A549 cells were transfected with 500 ng of pWRG7077-Empty, pWRG7077-HTNV-S, pWRG7077-HTNV-M for 24 h. As a control for inhibition of nuclear translocation, some cells were pretreated with MG132 for 2 h before stimulation with TNF-α. After incubation, cells were left untreated or treated with 50 ng/ml of TNF-α for 15 min and fixed and stained with antibodies against NF-κB p65 (green), HTNV N or Gc? proteins (red) and stained with DAPI to highlight nuclei (blue).

Fig. 4 Examination of TNF-α-induced IκBα degradation and NF-κB p50 and p65 levels in HTNV-S segment expressing cells. A549 cells were transfected with 500 ng of pWRG7077-Empty, pWRG7077-HTNV-S, or pWRG7077-HTNV-M for 24 h. To induce degradation of IκBα, cells were treated with 50 ng/ml of TNF-α for 15 min and lysates were prepared for immunoblotting. For un-induced samples, cells were left untreated. Proteins were transferred to PVDF membranes and probed with antibodies against IκBα, p50, p65, HTNV N protein, or GAPDH.

Fig. 5 Analysis of HTNV-N protein interaction with importin α proteins. A549 cells were co-transfected with 0 μg or 1.25 μg of importin α1-FLAG, importin α2-FLAG, importin α3-FLAG, or importin α4-FLAG and 1.25 μg 7077-Empty or 7077-HTNV-S encoding plasmid. Immunoprecipitations (IP) were performed 24 h posttransfection with anti-HTNV-N protein antibody bound to sepharose beads. Immunoprecipitates and whole-cell lysates (WCL) were analyzed by western blotting (WB) for expression of importin α proteins and HTNV N protein.

Fig. 6 **Examination of TNF-α-induced NF-κB p65 nuclear translocation and degradation of IκBα in HTNV-infected cells.** A549 cells were mock-infected or infected with HTNV at an MOI of 5. On day 5, mock- and HTNV-infected cells were left untreated or treated with 50 ng/ml of TNF-α for 15 min. (A) After fixation, cells were stained with antibodies against NF-κB p65 (green), HTNV N protein (red), and stained with DAPI to highlight nuclei (blue). Total cell lysates were prepared for immunoblotting and proteins were transferred to PVDF membranes. Blots were probed with antibodies against IκBα, p50, p65, HTNV N protein.

Fig. 1

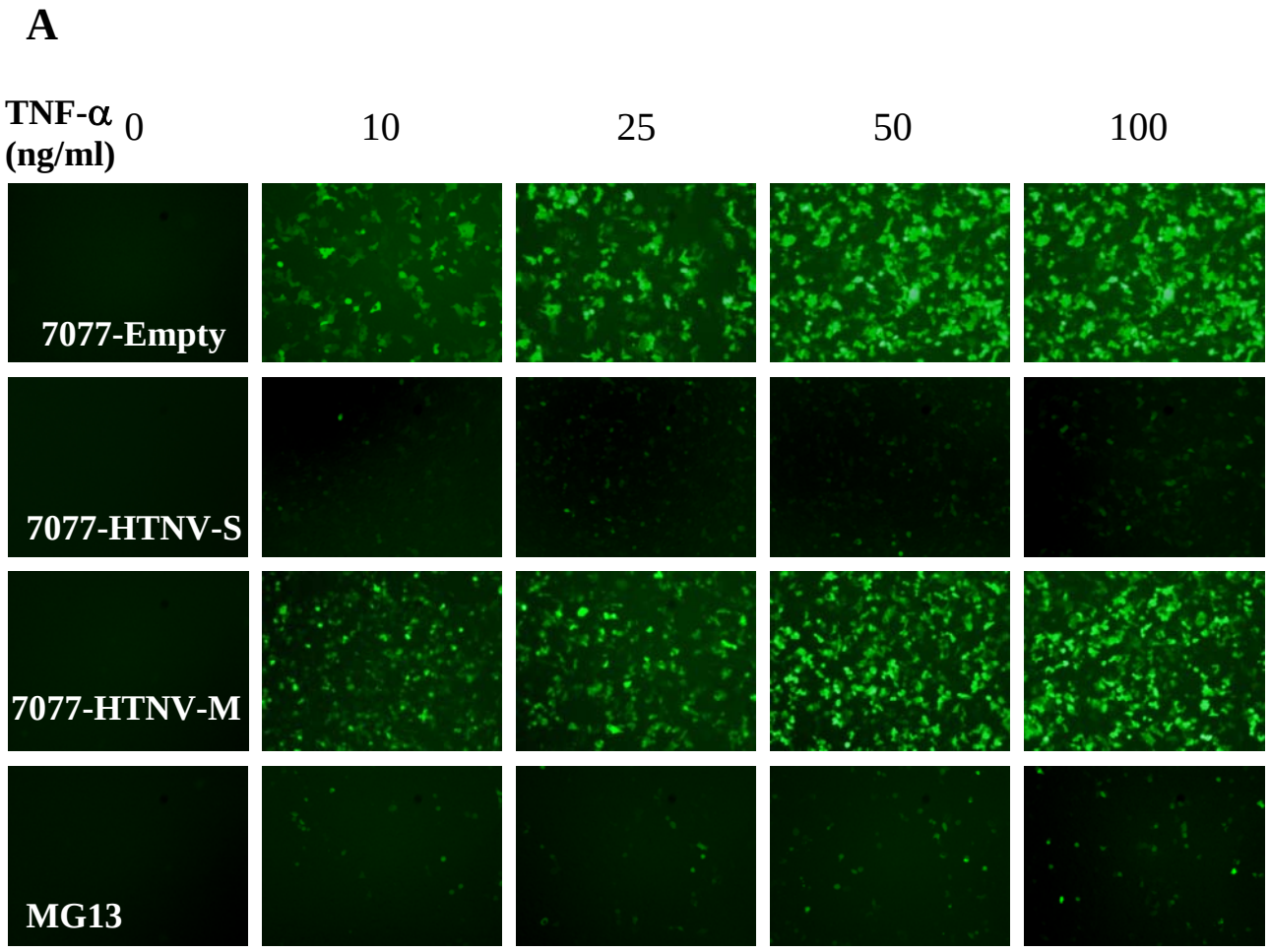

A
TNF-α (ng/ml)
0
10
25
50
100
7077-Empty
7077-HTNV-S
7077-HTNV-M
MG13

Fig. 1

B

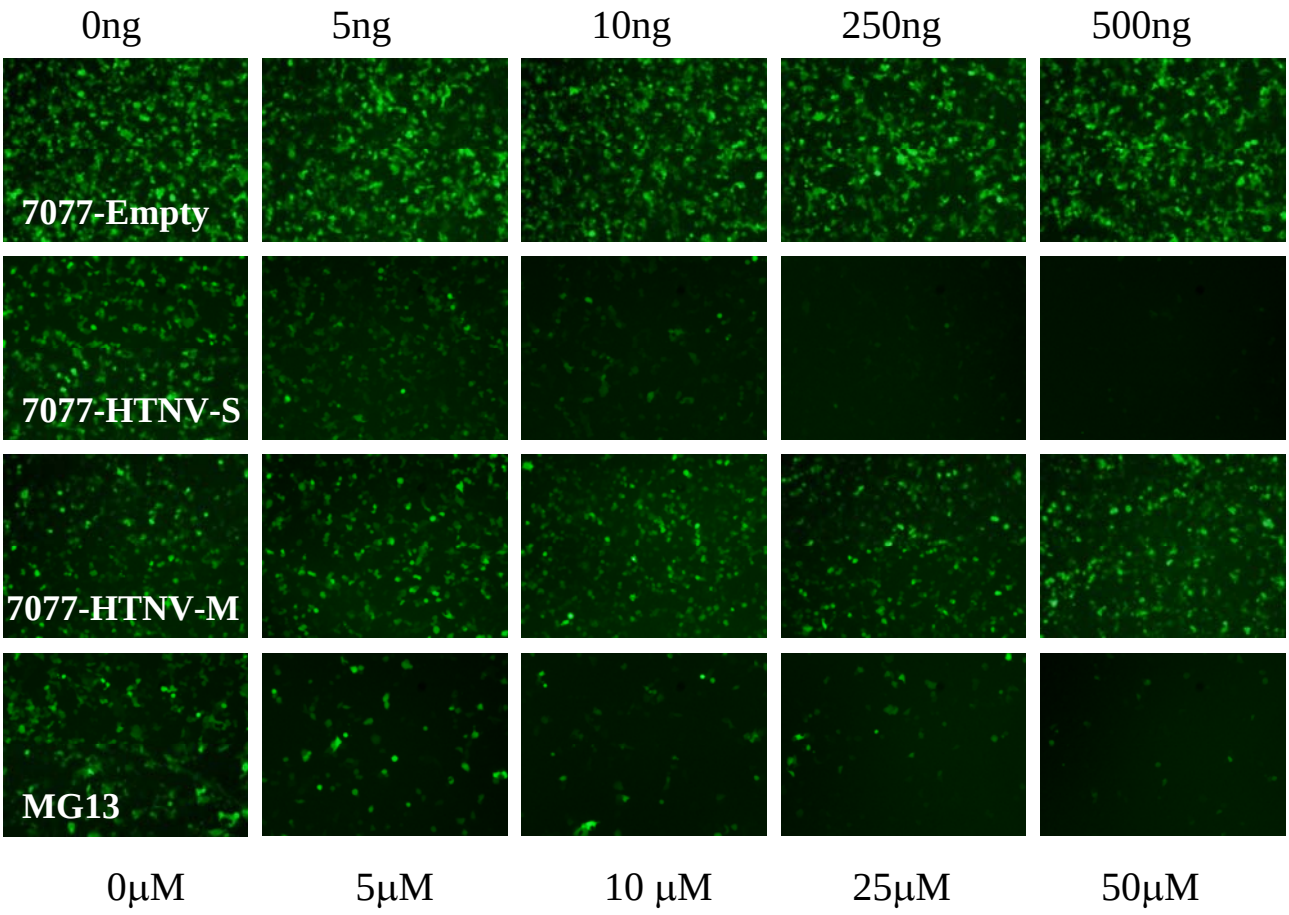
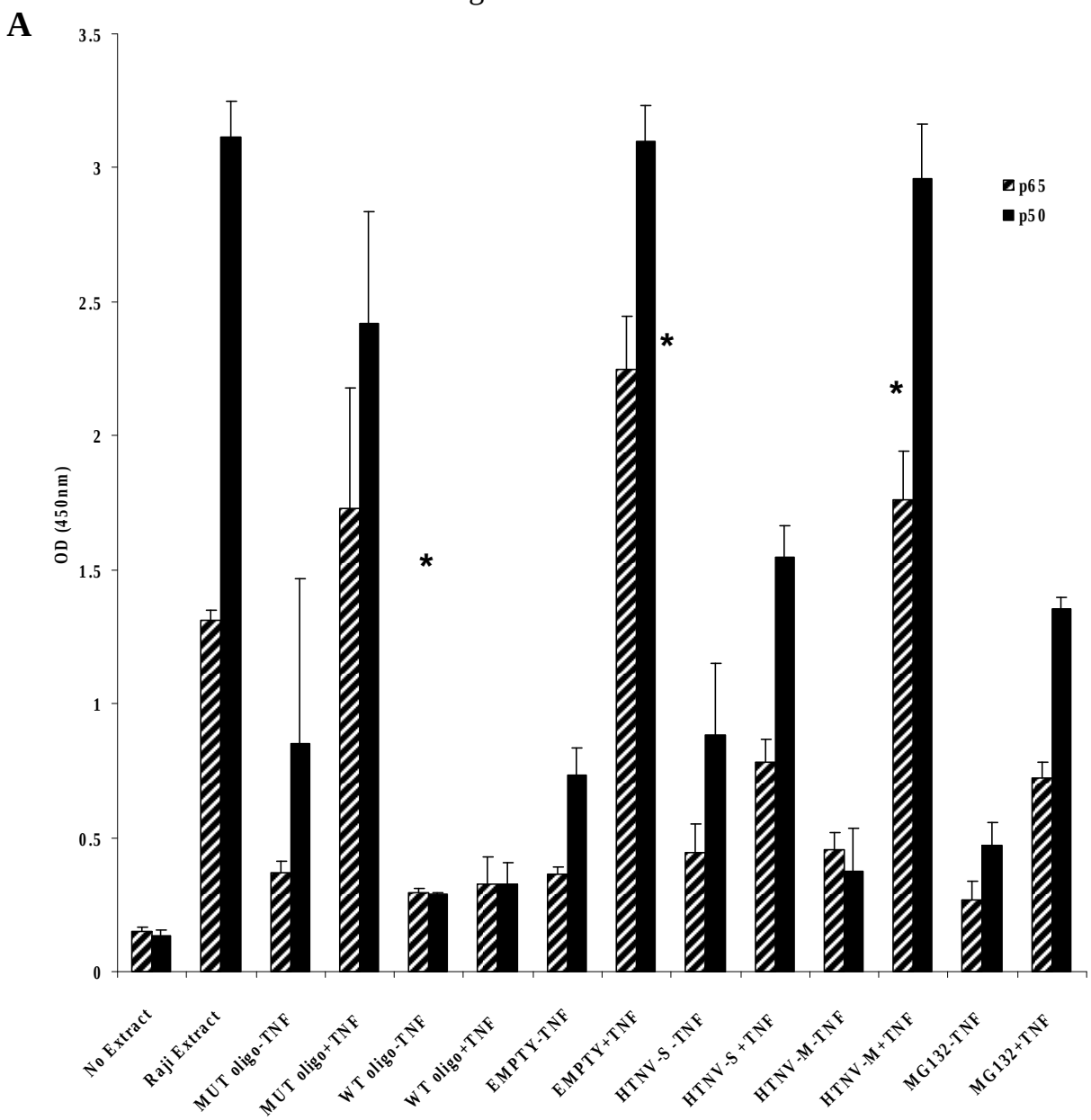

0ng
5ng
10ng
250ng
500ng
7077-Empty
7077-HTNV-S
7077-HTNV-M
MG13
0μM
5μM
10 μM
25μM
50μM

Fig. 2


A
3.5
3
2.5
2
1.5
1
0.5
0
OD (450nm)
p65
p50
*
*
*
No Extract
Raji Extract
MUT oligo-TNF
MUT oligo+TNF
WT oligo-TNF
WT oligo+TNF
EMPTY-TNF
EMPTY+TNF
HTNV-S -TNF
HTNV-S +TNF
HTNV-M-TNF
HTNV-M+TNF
MG132-TNF
MG132+TNF

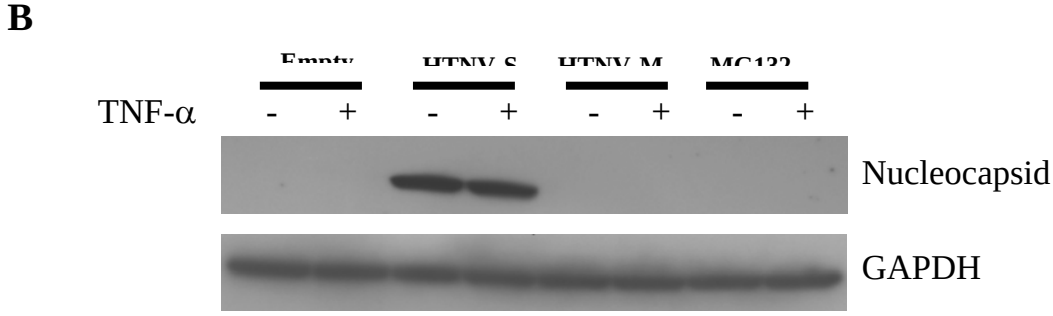

B
Empty
HTNV-S
HTNV-M
MG132
TNF-α
- + - + - + - +
Nucleocapsid
GAPDH

Fig. 3

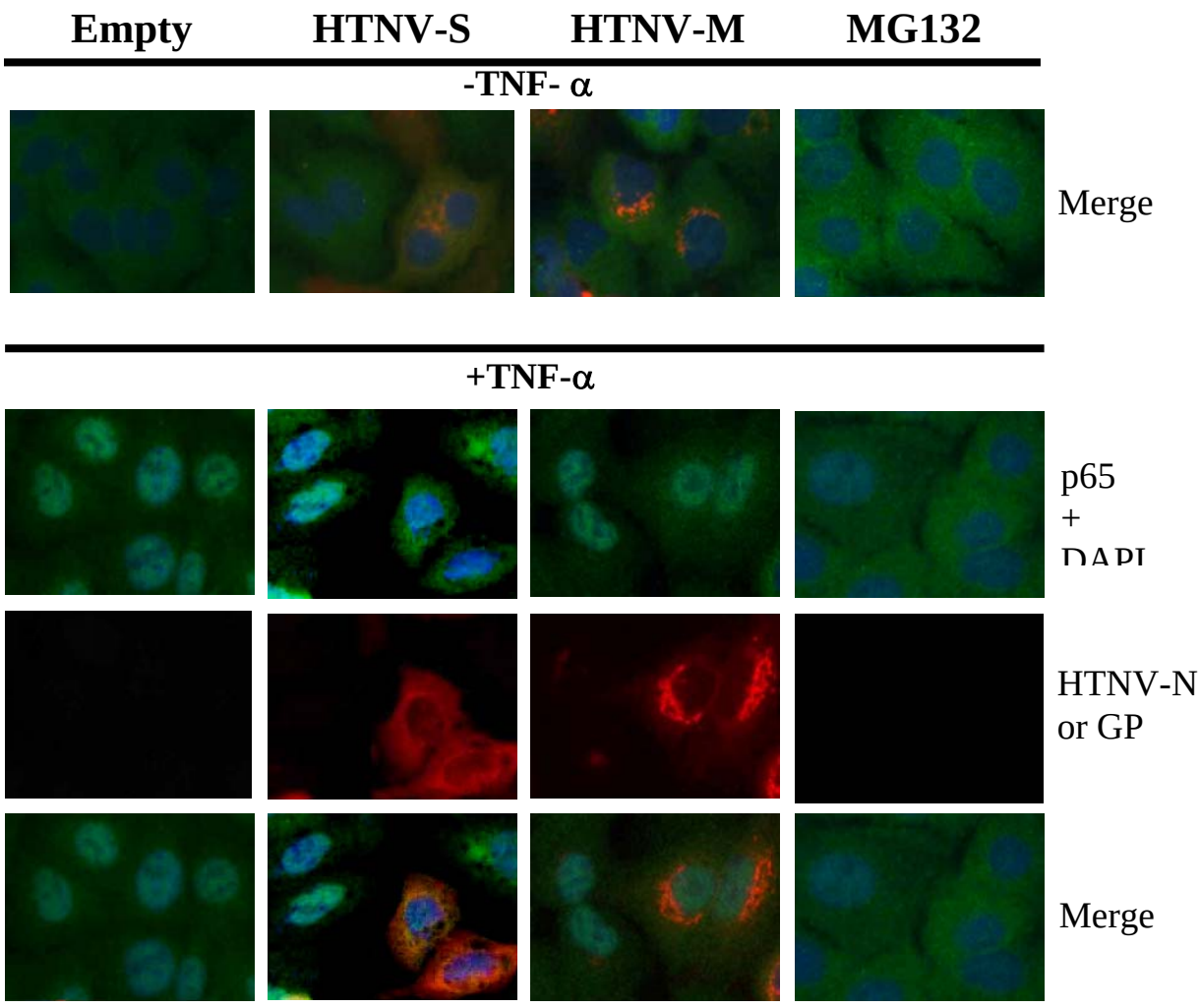

Empty
HTNV-S
HTNV-M
MG132
-TNF- α
+TNF-α
Merge
p65
+
DAPI
HTNV-N
or GP
Merge

Fig. 4

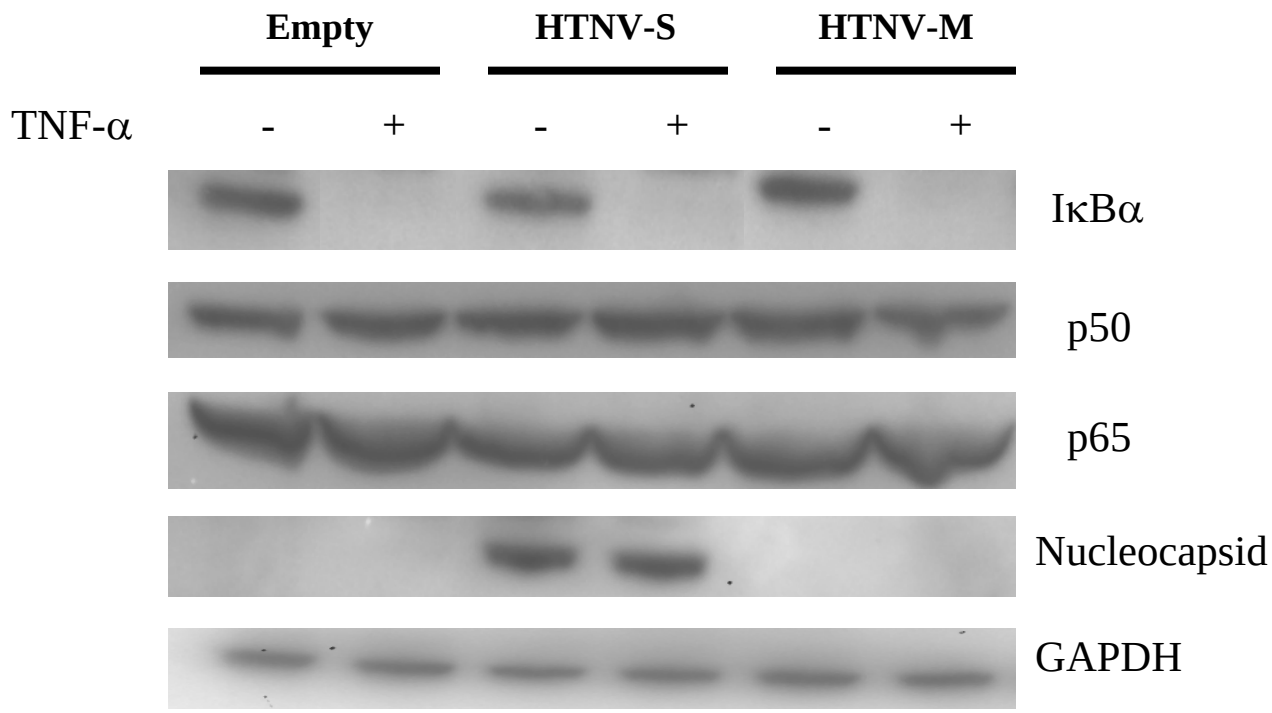


Fig. 5

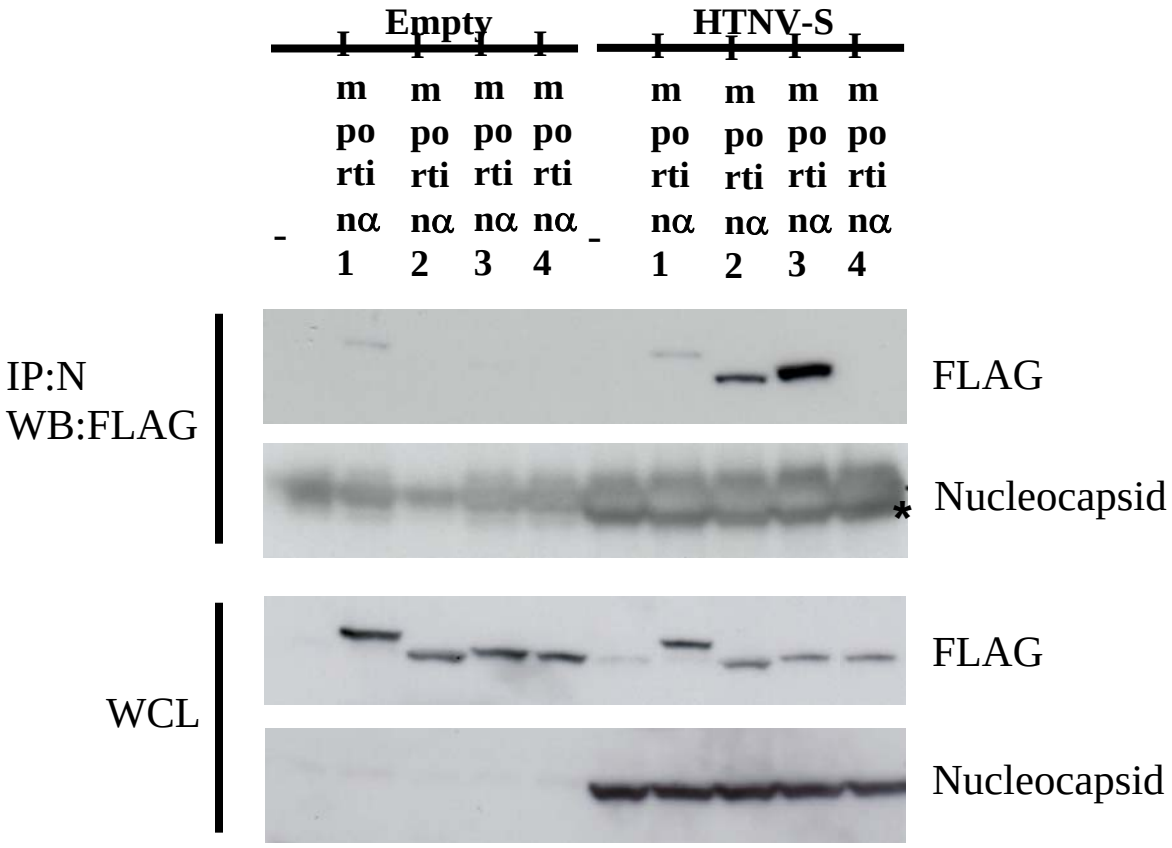

Empty
HTNV-S
-
mpo rti nα 1
mpo rti nα 2
mpo rti nα 3
mpo rti nα 4
-
mpo rti nα 1
mpo rti nα 2
mpo rti nα 3
mpo rti nα 4
IP:N
WB:FLAG
FLAG
Nucleocapsid
*
WCL
FLAG
Nucleocapsid

Fig. 6

A

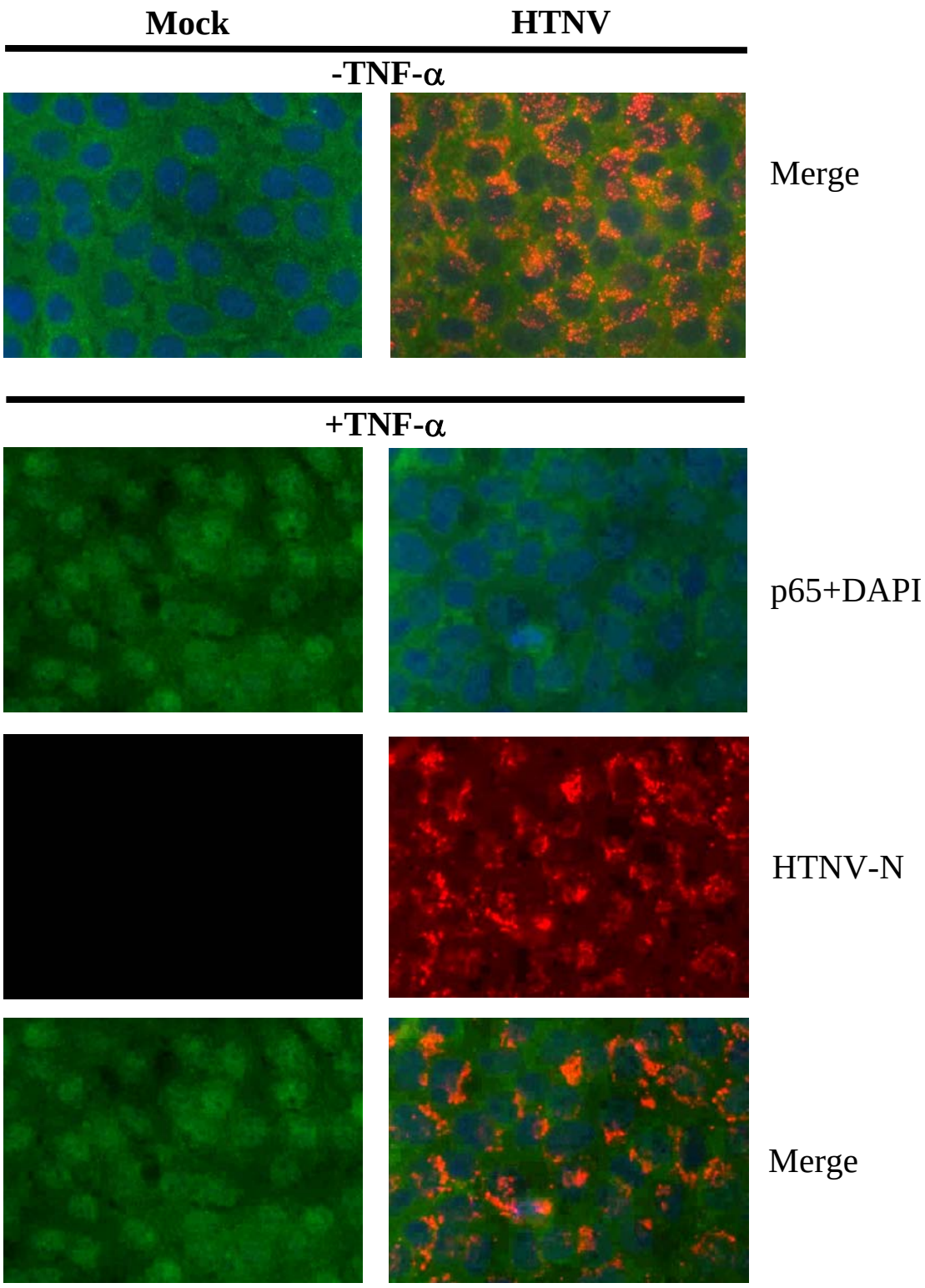


Fig. 6

B